Author's response:

We thank both reviewers for their comments and suggestions, which we addressed in the following individually. Reviewers' comments are given in black, while authors' responses are in red and changes in the manuscript in red italic.

Review #1

Dear authors,

This is a very valuable manuscript indicating the possible experimental artefacts in defining the isotope effects associated with different microbial pathways. It contains important new findings which provide better understanding of the observed variability in the isotope values typical for various denitrification pathways. Your in-depth experimental approach utilizing different incubation strategies unravel so far unknown mechanisms that govern the isotope signature of N2O.

No 1): The manuscript is mostly well structured and clear, I have only a few suggestions for structure enhancements. I am missing a data table with the original measured values, that could be placed in the supplementary information or as a repository file.

We have added a supplementary Excel file containing the datasets used to generate Figs. 3–9, including the individual time-resolved QCLAS measurements and the summary datasets used for the remaining figures. The data availability statement has been updated accordingly.

No 2): I think that very important point in this work is the responsible formulation of conclusions and assessment of applicability of these new results into the general definition of isotope characterisation of N2O denitrification pathways. How should we interpret these results? These are just 2 bacterial strains showing some unusual isotope values in very artificial experimental conditions, which are very distinct from natural environmental studies, where we deal with lower substrate availability, lower fluxes, microbes interactions, process limitations. The current ranges for N2O isotope mixing endmember for denitrification are based on controlled incubation experiments favouring denitrification, where quite homogenous isotope values have been reported for SP and d18O values (Toyoda et al., 2017, Lewicka-Szczebak et al, 2014, 2017, Yu et al., 2020). After figuring out that in natural soil incubations we mostly deal with near complete O-isotope exchange (Lewicka-Szczebak et al., 2016, Kool et al., 2007), indeed later pure culture studies showed large variations in this exchange between various microbial strains (Rohe et al., 2017, Haslun et al., 2018), however still maintaining differences between bacterial and fungal denitrification. Current findings arise further questions on this issue, but are the new values representative for typical conditions or are rather an experimental artefact? These new values must be surely taken into account by conducting experiments but do they have direct impact in the N2O analyses in natural soil and water environments?

The authors agree that the conclusions of this study should be framed carefully and that the implications for environmental isotope applications require a balanced discussion. We do not suggest that the elevated SP or variable $\delta^{18}O$ values observed here necessarily dominate under field conditions. Rather, our results demonstrate that physiological

state represents an additional source of variability that should be considered when interpreting isotope signatures of N_2O derived from denitrification. Accordingly, we do not advocate replacing isotopic signatures currently applied for identification with a single much broader interval of delta values. Instead, our findings support interpreting and, where appropriate, refining endmember ranges within the physiological and environmental context in which they were established. To better emphasize this point, we have added clarifying statements to Sect. 3.5 and the Conclusions.

We agree that laboratory incubation experiments cannot fully reproduce the complexity of natural environments, which is true for most, if not all, pure-culture studies that have contributed to the current understanding of N_2O isotope signatures. However, we do not agree that this limits their mechanistic relevance of our findings. The currently accepted denitrification endmembers are themselves derived largely from laboratory incubations and pure-culture studies. Our study demonstrates that even within this controlled experimental framework, isotopic expression varies substantially with physiological state and experimental design, indicating that endmember signatures are conditional rather than universal. Moreover, not all environmentally relevant N_2O-producing systems are characterized by low substrate availability and process limitation; examples include wastewater treatment systems, agricultural urine patches, and other N-rich environments. We therefore consider it plausible that the greater physiological and environmental complexity of natural systems may give rise to an even broader diversity of N_2O isotopic signatures. However, evaluating this hypothesis will require dedicated environmental studies.

To objectify results of our study we added some clarifying statements:

1. End Section 3.5 P25 L14: "*These findings do not imply that isotopic signatures currently used to identify N_2O produced by denitrification should be replaced by a single, much broader range of delta values. Rather, they demonstrate that physiological state of the microbial community should be considered explicitly when defining and applying isotopic signatures for environmental interpretation.*"

2. Conclusion P26 L11: *"Instead of defining and interpreting isotopic signatures as fixed constants, our results dermonstrate that they should be interpreted in the context of the physiological and environmental conditions under which they were established.*"

Specific comments:

No 3): Some publications cited in the manuscript are missing in the bibliography, eg: Denman et al., 2007, Lewicka-Szczebak et al., 2016, Ostrom et al., 2011, Ye et al., 1991,

Added.

No 4): P2, L 25-56 „and upstream processes" - unclear and a bit confusing, what these processes are.

We agree that the term "upstream processes" was too vague. We have revised the sentence to explicitly refer to the transformations preceding NO reduction and N–N bond formation. The revised text now reads (L2 P28):

"*In contrast, the oxygen isotopic composition of N_2O ($\delta^{18}O$–N_2O) reflects both NO reduction by NorB and the preceding transformations of nitrate and nitrite, and depends on the balance between nitrate-derived oxygen, nitrite–water exchange, and branching isotope effects.*"

No 5) P3, L3 I would add a problem on comparability of the experiments of pure culture studies and whole-soil or water communities. This seems to have a critical impact on the extent of O-exchange. While pure-culture studies show significant variations (Rohe et al., 2017, Haslun et al., 2018), whole-soil incubations favoring denitrification conditions typically show almost complete exchange (Lewicka-Szczebak et al., 2016; Kool et al., 2007). I suppose that the process rates, inter-microbial relations and low substrate concentrations in comparison to pure-culture studies play here a crucial role.

We agree that a variety of mechanisms could play a role in controlling O atom exchange between nitrite and water. The exact mechanism underlying why and how O atom exchange varies with physiological state is beyond the scope of this study and will require future targeted investigations. We also note that the whole-soil studies cited by the reviewer represent one class of environmental system but should not be considered representative of all natural denitrifying environments. Environmental N_2O isotopic signatures exhibit substantial variability across ecosystems, reflecting differences in microbial communities, substrate availability, hydrology, and environmental conditions (Li et al., 2026, https://doi.org/10.1029/2025GL116045).

In contrast to the reviewer statement, differences in O atom exchange between nitrite and water have actually been interpreted as an indication of NirS versus NirK activity (e.g., Gruber et al., 2022, https://doi.org/10.1016/j.wroa.2022.100130). Importantly, our results demonstrate that this interpretation is not justified, as O atom exchange also varies substantially with physiological state.

No 6): P4, L6 I think that the "resuspension assay" needs a bit more explanation. I suppose that you meant to resemble the bacterial denitrification method, but it may not be clear to all readers how it is performed, that the bacteria are grown, washed and placed in known nitrate solution, right? Please add some more details on this. And I would also add the information that this treatment is done as in usual bacterial denitrification approach (if I am right on that)

We agree that the term "resuspension assay" would benefit from a brief explanation. We have therefore added a short description of the workflow, indicating that cultures were first grown, then pelleted by centrifugation, and subsequently resuspended in a defined nitrate-containing medium prior to isotope analysis. We also explicitly state that this approach is adapted from the denitrifier method. The text has been modified to (L4 P12):

"CB-R denotes a resuspension assay in which actively grown cultures were harvested by centrifugation and resuspended in a defined nitrate-containing medium, following an approach adapted from the bacterial denitrifier method used to analyse the isotopic composition of nitrate (Sigman et al., 2001; Casciotti et al., 2002; Weigand et al., 2015)."

No 7): P5, L5 "pelleted cultures" – not clear to me, what this means, just lyophilized bacteria?

We agree that the term "pelleted cultures" may not be clear to all readers. We have therefore replaced it with a more explicit description indicating that the cultures were harvested by centrifugation before being resuspended in the defined nitrate medium. We modified the text accordingly (P5 L11):

"Second, for stationary-phase resuspension incubations (CB-R_aur_nat, CB-R_chlor_nat), cultures were harvested by centrifugation (4700 rpm, 10 min) and resuspended in a defined nitrate medium prepared in natural-isotope-abundance water."

No 8): P5, L7 "replaced KNO_3 with USGS32, USGS34, IAEA-N3" – this is not clear, which KNO3? In above sentence you say " defined nitrate medium", is it KNO3? What are USGS32, USGS34, IAEA-N3? Not every reader has to know, that you refer to isotope standards abbreviations. Better say sth like: "… was prepared in the same way as stationary-phase resuspension incubations but replacing the defined nitrate solution with international nitrate isotope standards usually applied for bacterial-denitrification analytical procedure (USGS32, USGS34, IAEA-N3)"

We agree that this description could be clearer for readers unfamiliar with the denitrifier method and nitrate isotope standards. We have revised the text to explicitly state that the nitrate substrate was replaced by international nitrate isotope reference standards and clarified the role of these standards. We modified the text to (L5 P35):

"*For CB-RS_aur and CB-RS_chlor (closed-batch resuspension assays converting nitrate isotope reference standards with* P. aureofaciens *and* P. chlororaphis, *respectively; experiment codes defined in Sect. 2.1.3 and Table 1), the same resuspension procedure was used, except that the nitrate substrate (20 nmol KNO_3) was replaced with 20 nmol of an international nitrate isotope reference standard (USGS32, USGS34, IAEA-N3, or ^{18}O-enriched IAEA-N3-spike).*"

No 9): P5, L10 I feel that integration of this sub-chapter with the previous one would increase the clarity and eliminate some of above questions and unnecessary repetitions – in this chapter 2.1.3 you do not only say about media but also explain how the experimental approaches were conducted. This chapter explains some above questions, integrating into one would save some repetitions.

We agree that the original subsection title no longer accurately reflected the content, as the section describes not only the media formulations but also key aspects of the resuspension procedure. Rather than merging the sections, we have revised the title of subsection 2.1.3 to "*Media composition and resuspension procedure*" to better reflect its content and improve readability.

No 10): P5, L17 "pelleted" – how the cultures were pelleted, with centrifugation?

We agree that the term "pelleted" may not be sufficiently clear to all readers. We have therefore replaced it with a more explicit description indicating that the cultures were harvested by centrifugation before resuspension. We now write (L5 P25):

"For stationary-phase resuspension incubations, actively grown cultures were harvested by centrifugation and resuspended in a simplified medium (Weigand et al., 2015) containing 7.5 mM NH_4^+ supplied as NH_4Cl, 30 g L^{-1} TSB, and 5 g L^{-1} K_2HPO_4 in the final solution."

No 11): P5, L22 "rather than to reproduce the exact conditions of the bacterial denitrifier method" – really? Why not? It is important comparison, and very needed, and your procedure is indeed the same as usually applied in the bacterial denitrification method, so I do not understand why you say this.

We agree that the previous wording could be misinterpreted. Our intention was not to imply that the resuspension assay differs fundamentally from the denitrifier method. Rather, we wished to distinguish between reproducing the biological resuspension approach and reproducing every analytical aspect of the denitrifier method. We have therefore revised the text to clarify that the resuspension protocol was adapted from the denitrifier method, while the headspace preparation was intended to standardize incubation conditions for our experimental objectives rather than to reproduce the complete analytical protocol. We modified the text as (P5 L31):

"The resuspension protocol was adapted from the bacterial denitrifier method,."

No 12): P6, L 13 I wonder what are your standards and how they were calibrated, I guess it must be self-produced standards, but the calibration of SP value of -93 permil is very challenging because normally the calibration scale is not so wide. How sure are you about this value? This is important because if this value is not perfectly right your isotope calibration may be significantly biased, since this point is far away from your samples. I wonder why you apply this strange standard? The commercially available medical N2O has typically the SP value near 0 per mil, and would be here a perfect addition to your STD1 and STD3. Please check how much your SP values will differ if you omit the STD2 from your normalization.

We appreciate the reviewer's concern regarding the use of STD2 and the assigned SP value. For clarification, the three N_2O reference gases (STD1, STD2 and STD3) used in this study are produced at Empa by mixing of isotopically pure N_2O gases with commercial N_2O. Their preparation and value assignment by Sakae Toyoda (Science Tokyo) follow the procedures described by Mohn et al. (2014, http://doi.org/10.1002/rcm.6982). Furthermore, the IRMS analytical framework established at Science Tokyo has been shown to be linear over a wide range of isotope values (Mohn et al., 2022, https://doi.org/10.1002/rcm.9296). We therefore anticipate low uncertainty in the assigned values of the applied N_2O isotope reference gases.

We agree with Reviewer #1 that the ^{15}N site-preference of STD2 is very artificial, i.e., not observed in nature. For GC-IRMS site-preference (SP) measurements, calibration is not carried out on SP itself. Instead, the assigned $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of the reference gases are used to calibrate individual isotope ratios after applying the ion-source scrambling correction and scale normalization. SP is subsequently calculated as $\delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$ (Kelly et al., 2023, https://doi.org/10.5194/bg-21-3215-2024). Thus, the calibration is fundamentally based on the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of the reference gases, not on their SP values.

With respect to $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, the use of the three reference gases spanning a wide range of isotopic compositions was intentional, and represents an advantage of the multi-point calibration approach, as it provides sufficient coverage to derive robust calibration factors. In addition, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of reference gases STD1-3 encompass the isotopic composition of most samples, which is considered good analytical practice. This would not be achievable using only STD1 and STD3, as suggested by Reviewer #1. Although such comprehensive calibration ranges are often unattainable in isotope studies because of the limited availability of reference materials, they are feasible here owing to the availability of STD1 – 3. Replacing STD2 with medical N_2O would narrow the calibrated $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ range, thereby weakening the calibration and increasing the uncertainty of the resulting calibration factors.

As an additional quality check, STD1 was routinely analyzed as an unknown sample in several analytical runs. The recovered isotope values agreed well with the assigned values (typically within ~1–2 ‰ for $\delta^{15}N^{\alpha}$, <1 ‰ for $\delta^{15}N^{\beta}$ and

$\delta^{15}N^{bulk}$, ~1–3 ‰ for SP, and ~1 ‰ for $\delta^{18}O$), providing an independent verification of the scrambling correction, scale normalization, and overall calobration procedure.

No 13): P7, L 12 – "N2O concentrations dropped below 50 ppm" – are you sure – 50 ppm is you limit for isotope analysis? Such huge concentration? (And later line 20)

The N_2O concentration chosen for isotopic analysis by QCLAS (50 ppm) does not represent a lower analytical threshold or detection limit but was selected to ensure optimal operation of both the experimental setup and the QCLAS analysis. Although lower concentrations (e.g. 5 ppm) could in principle be measured, doing so when reactor output concentrations are substantially higher (e.g. 300 ppm) would require extremely low sample gas flow rates during automated dilution. Furthermore, the direct absorption spectroscopy, as applied here, does not achieve the sensitivity of other spectroscopic approaches, such as ring-down spectroscopy (CRDS, Havsteen et al. 2026, https://doi.org/10.5194/amt-19-3557-2026), although those techniques saturate at higher concentrations (e.g. >1.5 ppm for CRDS). We have clarified this point in the revised manuscript to avoid potential misinterpretation by adding this statement (P8 L3):

"Choosing 50 ppm as target concentration for N_2O isotope analysis was a compromise between data coverage and response time of the experimental setup but does not represent a lower threshold of the analytics."

No 14): P10, L16 Regression line method to quantify O-exchange was applied in Snider et al., 2009 and Lewicka-Szczebak et al., 2016

Added.

No 15): P12, Fig.3 I think it would be clearer if the X-Axis was the same for all 3 panels, it can be simplified or complete Raileigh equation for all, the trends will be the same and equally visible.

We appreciate the reviewer's suggestion. However, the use of different x-axes is intentional because the three panels address different scientific questions. Panel A uses the Rayleigh coordinate to estimate the apparent ^{15}N enrichment factor applying a Rayleigh model. Panels B and C are not interpreted within a Rayleigh framework but instead show the evolution of SP and $\delta^{18}O–N_2O$ with reaction progress ($1 - f$). While using the same x-axis for all panels might improve visual consistency, it would obscure the intended interpretation, by suggesting the use of a Rayleigh model for both SP and $\delta^{18}O–N_2O$, which is not intended.

No 16): P13, L 9-11 This very large range of enrichment factors is very surprising, I would double check all the values and calculations, because on the graph the fractionation seems quite stable. Have you calculated with initial substrate isotope signature or have you taken into account the increasing delta value of nitrate? Note that for both approaches different equations should be applied (see eg. Denk et al, 2017 – Eq 9 and 10). Since in your experimental approach you are dealing with instantaneous product (delta pi) you need to calculate the instantaneous substrate isotopic signature (delta s) for each (delta pi). The last point with very low nitrate residual fraction may provide biased results, due to complete reaction, and should be disregarded from calculating mean values. It would be interesting to see the

graph, like Fig.3 with calculated epsilon values depending on the residual fraction. Your unique continuous data are here an excellent chance to investigate this dynamic. If the epsilon value is really so variable with residual fraction, the time-series of this change would be very interesting. Unfortunately, there are no original data given so that I could check the values myself.

We appreciate the reviewer's comments on this section. The enrichment factors and calculations have been checked and are correct. However, we believe there is a misunderstanding regarding how the enrichment factors were derived. In our study, the Rayleigh model analysis is applied to the cumulative isotopic composition of the accumulated N_2O product, following the cumulative-product approach described by Mariotti et al. (1981, https://doi.org/10.4141/cjss82-027) and Sutka et al. (2006, https://doi.org/10.1128/AEM.72.1.638-644.200). We do not estimate instantaneous isotope effects from the evolving substrate isotopic composition, as described by Denk et al. (2017, https://doi.org/10.1016/j.soilbio.2016.11.015). As we do not have temporally resolved $\delta^{15}N\text{-}NO_3^-$ data, we are unable to apply the mathematical framework suggested by the reviewer to the presented dataset.

We agree, however, that this distinction could be explained more clearly in the manuscript. We have therefore revised the beginning of Sect. 3.2 and the legend of Fig. 3 to explicitly state that the reported ε values are apparent enrichment factors derived from the cumulative N_2O product and to clarify the rationale for using this Rayleigh framework.

Changes made:

1. Beginning of Sect. 3.2 (P14 L3): "*... using the Rayleigh equation as presented in Mariotti et al. (1981) and Sutka et al. (2006) (Fig. 3A). The isotopic composition of the accumulated reaction product N_2O was calculated from the amount and isotopic composition of instantaneously formed and analysed N_2Ot.*"
2. Applicability pharagraph (P14 L25): *"Rather, the Rayleigh analysis is used as a first-order framework to derive apparent $\varepsilon^{15}N$ values from the cumulative N_2O product pool [...]*"
3. Literature comparison (P14 L30): "*This dataset, largely derived from Denk et al. (2017), includes enrichment factors determined under diverse experimental conditions and using different formulations of the Rayleigh model applied to either the instantaneous or accumulated product, or to the substrate. Nonetheless these enrichment factors provide a context for comparing the magnitude of isotope fractionation observed in the present study.*"
4. Fig. 4 (P15 L6): "*[...] which integrates enrichment factors derived from different experimental systems and using different mathematical formulations (e.g., soils, aqueous environments, and pure-culture incubations) [...]*"

No 17): P18, L 10-13 What value do you assume for branching? For equilibrated you used 10-15 permil (equilibrated nitrite – N2O) and for no-equilibration it makes 38 permil (nitrate-N2O). Is this difference due to additional effect by the nitrate-nitrite reduction step? I think you should provide values of branching effect that you assume here and any citation for these.

We thank the reviewer for this helpful suggestion. We agree that the fractionation factors underlying our conceptual framework were not stated explicitly, which may have caused confusion. We have therefore revised the manuscript to specify that the O-isotope fractionation factors assumed in our interpretation follow Casciotti et al. (2002, https://doi.org/10.1021/ac020113w) and Casciotti et al. (2007, https://doi.org/10.1021/ac061598h). This includes the approximate 25–30 ‰ difference in $\delta^{18}O$ between nitrate and nitrite and the approximately 10–15 ‰ branching isotope effect associated with the reduction of NO_2^- to N_2O. We have also revised the figure legend to specify that the

expected $\delta^{18}O–N_2O$ range under complete nitrite–water equilibration assumes this branching isotope effects and have added the corresponding references.

Modifications made:

5. Fig 6 (P19 L2): "*The shaded red band (+14.5 to +19.5 ‰) represents the expected $\delta^{18}O–N_2O$ range assuming complete oxygen atom equilibration between nitrite and water and a branching isotope effect of approximately 10–15 ‰ (Casciotti et al., 2002; 2007)*"
6. Scenario introduction (P19 L14):" *To interpret the observed variability in $\delta^{18}O–N_2O$, three reference scenarios can be defined. Following the framework of Casciotti et al. (2002; 2007)), nitrate reduction to nitrite is associated with an oxygen isotope fractionation of approximately 25–30 ‰, while NO_2^- reduction to N_2O is associated with a branching isotope effect of approximately 10–15 ‰. In the absence of oxygen atom exchange between nitrite and water, $\delta^{18}O–N_2O$ is therefore expected to be approximately 50 ‰, reflecting the nitrate-source signature ($\delta^{18}O–NO_3^-$ = +12.4 ± 0.3 ‰) shifted by the combined isotope effects described above.*"

No 18): P21, L37-38 This statement actually only refers to *P. aureofaciens*. *P. chlororaphis* shows absolutely stable O-exchange across all experimental conditions. From your results I would say that rather high exchange is typical for these both strains but for *P.aureofaciens* the resuspension experiments create specific conditions that massively limit the O-exchange.

We agree that the marked influence of incubation conditions on O atom exchange between nitrite and water was observed primarily for *P. aureofaciens*, whereas *P. chlororaphis* exhibited consistently high O atom exchange across the experimental conditions investigated. We have revised the text accordingly to better reflect the distinct responses of the two strains and avoid overgeneralizing this observation.

We added in P. 22 an additional sentence: "I*n contrast, P. chlororaphis maintained consistently high oxygen atom exchange across the experimental conditions investigated.*"

No 19): P22, L20 Any conclusions why such differences are possible? For P.aureofaciens – from complete exchange in closed batch incubation to almost no exchange in resuspension experiments. These approaches are not so much different… was it the incubation time that differs? Is it the period of no nitrate available for bacteria in resuspension method for 24 hours – after this "hungry" period nitrate is rapidly consumed and therefore exchange is so low? Any conclusion here what the governing difference between these approaches could be ?

We thank the reviewer for this insightful comment. We agree that the mechanistic basis for the contrasting oxygen isotope exchange between nitrite and water observed under active-growth and resuspension conditions deserves further discussion. We have expanded the Discussion to include a mechanistic interpretation based on nitrite intermediate dynamics. During active growth, nitrate reduction, nitrite reduction, biomass synthesis, and enzyme expression occur simultaneously, which may prolong the residence time of nitrite (or downstream oxygen-bearing intermediates) in the aqueous phase and increase the opportunity for oxygen atom exchange with water. In contrast, resuspension assays employ fully induced cells that rapidly reduce the supplied nitrate, likely shortening the residence time of nitrite and thereby limiting oxygen atom exchange. We further note that additional factors, such as pH-dependent exchange

kinetics or other physiological differences between growth states, may also contribute to the observed patterns. However, these effects cannotr not be resolved with the present dataset and therefore represent an important topic for future investigation.

We have added these aspects to the paragraph in question (P24 L16): *"A mechanistic explanation for this variability likely lies in the dynamics of the nitrite intermediate pool (Casciotti et al., 2007). Oxygen exchange between nitrite and water requires sufficient residence time of NO_2^- to approach isotopic equilibration. During active growth, differences in nitrate reduction rates, nitrite accumulation, and consumption can alter both the size and lifetime of this intermediate pool, thereby modulating the extent of exchange. In addition, because actively growing cultures simultaneously undergo biomass synthesis and enzyme expression, nitrate reduction and downstream nitrite reduction may be less tightly coupled than in resuspension assays, allowing nitrite to persist longer before reduction. In contrast, under resuspension conditions, fully induced cells rapidly reduce the supplied nitrate, and tighter coupling between nitrate and nitrite reduction likely limits nitrite accumulation and, consequently, oxyygen isotope exchange. Additional physiological factors, such as pH-dependent exchange kinetics (Buchwald and Casciotti, 2013) or changes in the cellular microenvironment, might also contribute but could not be resolved in the present study and therefore warrant future investigation. These observations indicate that oxygen exchange reflects system-level metabolic dynamics rather than fixed enzymatic behavior."*

No 20): P22, L22 Also in Rohe et al., 2017

Added.

No 21): P23, Conclusion section

In this section it is very important to reasonably asses the significance of your findings into general field application of N2O isotopes in pathways partitioning. Should we increase the typical SP-denitrification values with all possibly SP values demonstrated here? These are probably very rare cases in natural conditions (if ever applicable) and would actually make the isotope N2O source -partitioning useless, because SP-ranges of nitrification and denitrification would overlap. It is reasonable to take these results into account? Which conditions in natural field studies could possibly enhance the untypical isotope values for N2O originating from denitrification.

We agree that the implications of our findings for environmental isotope applications deserve careful discussion. However, we do not think the available evidence supports the assumption that the elevated SP values observed here are necessarily rare or negligible under natural conditions. Our experiments demonstrate that denitrification itself can produce a substantially broader isotopic range than is generally assumed under specific physiological conditions. Together with the recent observations of Wang et al. (2024, https://doi.org/10.1073/pnas.2319960121), these results indicate that elevated SP values are an intrinsic property of denitrification under certain physiological states rather than an analytical artifact.

Our study was not designed to determine how frequently such physiological states occur in environmental systems. Consequently, we consider both the assumption that they are common and the assumption that they are rare to be premature. Instead, our results identify physiological state as an additional source of variability that should be considered when interpreting N_2O isotopocule data.

We therefore do not propose indiscriminately broadening existing denitrification endmember ranges indiscriminately. Rather, our findings suggest that these endmembers should be interpreted within the physiological and environmental context in which they were established. Our results expand the known isotopic space that denitrification can occupy under specific physiological conditions and demonstrate that applying fixed endmember values across fundamentally different physiological states may represent an oversimplification. The recent work of Strubbe et al. (2026, https://doi.org/10.1016/j.watres.2026.125580) illustrates how process endmembers can instead be constrained for the environmental system under investigation.

We have added this sentence in the conclusion (P26, L11): "*Rather than defining and interpreting isotopic signatures as fixed constants, our results support consideration of the physiological and environmental conditions under which they were established.*"

No 22): Are the elevated SP values at the beginning of the experiment not just an experimental artefact with no real impact on the real condition isotope characteristics? The initial experimental condition - before an equilibrium is established - show very distinct values – for all isotope values. Are these not very temporary values which do not occur when we deal with constant dynamic equilibrium conditions in natural environments?

The elevated SP observed at the beginning of the experiments were reproducibly observed across independent experiments, making an analytical or experimental artefact unlikely. Conventional closed-batch incubations measure the isotopic composition of the cumulative N_2O pool present in the headspace. Even when sampled shortly after the onset of N_2O production, the measured isotope signature represents the integrated contribution of all N_2O produced up to that point rather than the instantaneous isotopic composition of newly formed N_2O. In contrast, our continuous flow-through approach resolves the isotopic composition of N_2O as it is produced, allowing transient early-stage isotopic dynamics to be observed directly.

The reviewer's comment further assumes that environmental denitrification generally occurs under steady-state conditions, such that these transient signatures would not be relevant. However, environmental systems are inherently dynamic, with fluctuating substrate availability, redox conditions, and microbial activity, such that steady-state conditions often cannot be assumed. There is currently no evidence that transient high-SP denitrification signatures do not occur in nature. In fact, elevated SP values observed in environmental studies are frequently attributed to nitrification or other pathways because they fall outside the canonical denitrification endmember range. Our results demonstrate that denitrification itself can produce substantially higher SP values, consistent with the findings of Wang et al. (2024, https://doi.org/10.1073/pnas.2319960121). Whether, and under which environmental conditions, these transient? signatures contribute to observed N_2O isotopic compositions remains an important question for future research.

No 23): P24, L2-4 Please publish your original data in accessible form in public repository. These are very important and complex measurements and possibly other researchers may need to perform further calculations with the data, eg. to add your findings into the common literature database on isotope fractionation factors.

From the dataset that you present in your current manuscript, the check of your calculated isotope effects is not possible – e.g. I tried to roughly check the epsilon15N values that you referred, and this is not possible with the available data. Please make sure that all the needed values for the calculation are provided in the repository files.

The dataset will be made available, as described in our updated Data Availability Statement.

Review #2

Summary

This study presents an examination of N_2O production by two denitrifying bacterial strains lacking nitrous reductase. The study tests several of the assumptions about the nature of N_2O production signals deduced from isotopes of N_2O in a number of useful experimental contexts, including both closed and open type systems and using both laser based and mass spectrometric analytical tools. By analyzing the product N_2O continuously over the course of nitrate consumption, the authors demonstrate that N_2O site preference is not constant, but rather shifts over the course of the culture's consumption of nitrate. The authors also examine the nature of O isotopes in product N_2O, demonstrating that growth status (exponential vs stationary phase) and growth conditions (closed bottle vs gas flushed system) have strong impacts on the degree of O atom exchange with water and hence the resulting $d^{18}O$ of the N_2O. While the study largely examines these two cultures in the context of their utility in the very popular denitrifier method, they demonstrate that these large shifts and deviations do not appear when following the classic method protocols. Rather, their results more importantly point to a wider range of expected N_2O compositions from processes (such as denitrification) that stem from physiological changes in the bacteria themselves – dynamics that may easily occur under a wide range of natural settings. This type of exploration of microbial physiological state and its impact on isotope expression is critical and sorely lacking in our current understanding of isotope dynamics and its extension to field studies. The study does a great job of demonstrating impacts of growth states and conditions on the physiological expression of isotope effects.

Overall, the paper is a very thorough and interesting exploration of microbial metabolic products and how they are influenced by different growth conditions. This study has important implications for environmental studies and delves into relevant aspects that are understudied in studies of environmental systems.

No 24): A final note - the paper could probably be shortened by ~10% without any loss of content. There are some repetitive aspects throughout.

We thank the reviewer for this suggestion. We have carefully reviewed the manuscript to reduce unnecessary repetition and improve conciseness where possible. However, we have retained the overall level of detail, as we believe it is important for communicating the methodological approach and the interpretation of the results.

Major Comments

No 25): My biggest question/concern is regarding the use of a closed system accumulated product framework for interpreting the 'gas-flushed' experiments (described in Section 2.2.3). Figure 2B shows that N_2O levels in the bottle were transient (e.g., continuously flushed). If N_2O really is continuously swept out of the culture and analyzed by QCLDAS, then these measurements would represent point measurements of the 'instantaneous product.' I am confused about how the Rayleigh accumulated product approach is applied to this open type of analytical system. Perhaps the authors are mathematically 'accumulating' the N_2O in a mass balance manner, such that the evolving N_2O composition truly does represent the integrated accumulating product N_2O. But if so, then this was lost on me and

should be clarified. If this is not the case then I am confused about what is being shown in Figure 3A. The authors clearly understand the dynamics here as they describe 'conventional accumulated product' calculations in other portions of the paper. If they truly are measuring the instantaneous product, then the regression of $d^{15}N_{bulk}$ of N_2O should be linearized against -ln(f). If the instantaneous product is regressed against (-f * ln (f))/(1-f), the plot will look quasi-linear (as in Figure 3A), but the slope would not be equivalent to the isotope effect in this case. It would be much higher. Note that in the individual experiments shown (13 – 19) in the Appendix B – the curvature of $d^{15}N$ is very clear toward the end – as would be expected. As such, I'm not sure the isotope effects reported on P13 L9-13 are correct. But it is also possible I have misunderstood. Either way I think there needs to be some clarity here.

We thank the reviewer for carefully identifying this point. The gas-flushed system indeed measures the instantaneous N_2O leaving the reactor. However, the Rayleigh analysis is not based directly on these individual QCLAS measurements. Instead, cumulative N_2O production was reconstructed by integrating the FTIR-derived N_2O flux over time using the known carrier-gas flow. The cumulative N_2O production was then used in a mass balance approach to estimate the remaining nitrate fraction (*f*) and calculate the isotopic composition of the cumulative N_2O product pool. Accordingly, the results presented in Fig. 3A represent the accumulated product pool rather than instantaneous QCLAS measurements plotted against the Rayleigh model coordinate.

We agree that this distinction was not sufficiently clear in the original manuscript. Although the cumulative-product formalism is described in Sect. 2.2.3, we recognize that it should also be reiterated where the Rayleigh analysis is presented. We have therefore clarified this point at the beginning of Sect. 3.2 and in the legend of Fig. 3 by explicitly stating that the reported ε values are derived from the reconstructed cumulative N_2O product pool using the cumulative-product formulation of Mariotti et al. (1981, https://doi.org/ 10.1007/bf02374138) and Sutka et al. (2006, https://10.1128/aem.72.1.638-644.2006) (see also our response to Reviewer #1, Comment 16).

No 26): How widely has the notion of NirS and NirK imparting differing O atom exchange dynamics really been assumed and/or applied in other studies? I understand that this is an important outcome of your study, but don't necessarily feel that this distinction between NirS and NirK is truly 'canonical' as described in the paper numerous times. I would recommend toning this down a little bit maybe?

We thank the reviewer for this helpful comment. We agree that we overstated how firmly established the distinction between NirK- and NirS-bearing denitrifiers is by referring to it as "canonical" throughout the manuscript. Although the literature has repeatedly proposed that NirK and NirS differ in nitrite-water oxygen atom exchange behaviour, we agree that this should be regarded as a widely discussed hypothesis rather than a universally accepted paradigm. We have therefore revised the terminology throughout the manuscript while retaining our central conclusion that oxygen atom exchange is not determined solely by nitrite reductase identity.

Modifications made:

7. Section 3.5 (P23 L36): Replaced *canonical* with *characteristic* and revised the text to refer to $\delta^{18}O–N_2O$ signatures commonly associated with NirK and NirS.

8. Introduction: Revised (P2 L38) "*This contrast has led to the widespread assumption...*" to "*This contrast has often been interpreted as evidence that NirS and NirK differ systematically in their influence on $\delta^{18}O$–N_2O expression (Kool et al., 2007; Guber et al., 2022).*"
9. Introduction: Revised (P3 L31) "*...including the assumed NirK/NirS control on oxygen atom exchange with water...*" to "*...including the proposed relationship between nitrite reductase identity (NirK/NirS) and oxygen atom exchange with water...*"

No 27): Did the GasMet FTIR Analyzer ever see any NO? Were there any variations in NO dynamics that could be connected with accumulation of intermediates or dynamics of N_2O SP, for example? P16 L8.

We thank the reviewer for this interesting question. Although the Gasmet FTIR analyzer was configured to monitor NO, measured concentrations remained at or below the instrument detection limit (0.13 ppm) throughout the experiments. Consequently, we were unable to obtain sufficiently resolved NO time series to evaluate relationships between transient NO accumulation, intermediate dynamics, and the observed SP evolution. Such measurements would indeed be valuable, particularly in light of the proposed involvement of alternative NO reductases, and represent an interesting direction for future work.

No 28): Is there any speculation about how these growth states and conditions could be impacting intracellular pH (which of course has a big impact on abiotic NO_2^- -water equilibration rates)? With respect to nitrite-water exchange in general – is this considered to be abiotic or enzyme-catalyzed and why?

We thank the reviewer for this insightful comment. This point substantially overlaps with Reviewer #1, Comment 19. We have therefore expanded the Discussion to include a mechanistic explanation based on differences in nitrite residence time between active-growth and resuspension conditions. We also note that additional factors, including pH-dependent exchange kinetics, may contribute but cannot be resolved with the current data set, and therefore warrant future investigation (see also our response to Reviewer #1, Comment 19).

I enjoyed the discussion about alternative NOR enzymes that could be involved!

Minor Comments

No 29): P1 L23: perhaps use 'suggesting' instead of 'indicating?'

Agreed. "Suggesting" is probably more appropriate throughout when discussing mechanistic interpretations.

No 30): P1 L35: "physiological state and metabolic context" – could you be a little bit more specific? In the paper this is explored in more detail obviously, but it could be helpful to mention what specific aspects are being investigated.

We agree that the phrase "physiological state and metabolic context" is rather general in the abstract. We have therefore revised the sentence to provide more specific examples, by explicitly referring to differences between active-growth and stationary-phase resuspension conditions and associated differences in nitrite turnover.

We have added to the sentence (P1 L36): "*[...], including active growth versus stationary-phase resuspension and differences in nitrite turnover.*"

No 31): P7 L11: extraneous period

Corrected.

No 32): P10 L 29: Do the low yields imply that the culture died? NO_3^- was not completely consumed? Or some other product accumulated? Were the cultures not happy with the continuous N2 bubbling?

We do not think that the lower N_2O yields imply a loss of culture viability. Rather, they indicate that N_2O was not produced quantitatively from the supplied nitrate. The missing fraction may reflect incomplete nitrate consumption and/or the formation of other reduced nitrogen products that were not quantified in this study. This also represents a limitation of the Rayleigh mass balance approach. In the gas-flushed setup, the residual nitrate fraction (*f*) is reconstructed from cumulative N_2O production relative to the initial nitrate addition, rather than from direct measurements of residual nitrate. Consequently, *f* represents an N_2O-based approximation rather than a direct determination of the remaining nitrate. We have clarified this limitation in the manuscript. Although we cannot exclude the possibility that continuous N_2 stripping imposed some physiological stress on the cultures, we have no experimental evidence to evaluate this possibility.

We have added two more elements in Section 3.1:

10. P11 L14: *"Accordingly, cumulative N_2O yields indicated efficient denitrification (Fig. 2A)., although they varied between incubations, indicating that N_2O was not always the sole nitrogen oxide produced (Appendix B, Fig. B1).*"
11. P11 L19: *"These results demonstrate that both incubation approaches supported efficient denitrification and provided well-constrained systems for isotopic interpretation.*"

No 33): Figure 2: what is the timescale? Days?

Yes, days. We have added the missing information to the figure's legend.

No 34): P17 L10: I think I would disagree that there was comparatively 'little sensitivity' in the P. chlororaphis culture, they still shifted by 10-20 ‰.

We agree that the wording "comparatively little sensitivity" understated the observed response of *P. chlororaphis*. Although the shifts in $\delta^{18}O–N_2O$ were substantially smaller than those observed for *P. aureofaciens*, they still amounted to approximately 10–20 ‰ between incubation modes. We have therefore revised the text to state that *P. chlororaphis* exhibited a less pronounced response to incubation configuration while maintaining consistently elevated $\delta^{18}O–N_2O$ values.

We modified the text as (P16 L13): "*These contrasts indicate a strong incubation-mode sensitivity in P. aureofaciens, whereas P. chlororaphis maintained consistently elevated $\delta^{18}O–N_2O$ values with a less pronounced response to incubation configuration.*"

No 35): Figure 6: For the GF cultures – are these endpoint measurements – or mass integrated $d^{18}O$ values? For oxygen isotopes of N_2O, does this actually matter? In theory, all else being constant, the $d^{18}O$ of N_2O should hold steady (and it doesn't as noted in P. aureofaciens Fig 3C). But here in Figure 6 the 'final' values of N_2O are indicated (single time point) and compared with closed batch values – which are integrated values of the entire reaction. It feels a bit like comparing apples to oranges, so to speak. Still useful, but there are nuanced differences.

We thank the reviewer for pointing out this distinction. The gas-flushed values shown in Fig. 6A represent the final $\delta^{18}O$–N_2O values measured at the end of the time-resolved incubations, whereas the closed-batch values represent the isotopic composition of the accumulated N_2O pool. Thus, the comparison is not between mathematically equivalent quantities. Nevertheless, we believe it remains informative because it illustrates the contrasting $\delta^{18}O$–N_2O signatures produced under the two incubation modes. To avoid confusion, we have clarified in the figure legend that the gas-flushed values represent endpoint measurements from the time-resolved experiments, whereas the closed-batch values represent the accumulated N2O product pool.

No 36): P18 L12: What is the value used for this branching effect and how was it determined (35‰)? Note it might be useful to remind readers that there are three individual branching effects that impact N_2O (branching during NO_3^- reduction, during NO_2^- reduction and during NO reduction). Also has been referred to as "O atom abstraction" isotope effects. For example P19 L19 – "the intercept approximates the sum of the nitrite-water equilibrium isotope effect and the branching isotope effect." – here I think we are considering the 2nd and 3rd downstream branching isotope effects, rather than the 1st effect at NO_3^- reduction. Some clarification would be good for the reader.

We thank the reviewer for this helpful comment. This point substantially overlaps with Reviewer #1, Comment 17, and we agree that the oxygen isotope accounting underlying our conceptual framework should be stated more explicitly. We have therefore revised the manuscript to clarify that the expected $\delta^{18}O$–N_2O values reflect the combined oxygen isotope effects associated with nitrate reduction to nitrite (approximately 25–30 ‰) and the branching isotope effect during NO_2^- reduction to N_2O (approximately 10–15 ‰), following Casciotti et al. (2002, http://doi.org/10.1021/ac020113w) and Casciotti, et al., 2007 (https://doi.org/10.1021/ac061598). We have also revised the terminology throughout the manuscript to explicitly distinguish these two contributions and updated the figure legend accordingly (see also our response to Reviewer #1, Comment 17).

No 37): P18 L36 - unnecessary comma after 'values.'

Removed.

No 38): Figure 9 - I'm not sure this figure is critical for the paper if space is required. I feel like the point is already well made in the corresponding text.

We thank the reviewer for this suggestion. We agree that the key conclusions are presented in the text. However, we have chosen to retain Fig. 9 because it provides a concise visual synthesis of the study's main conceptual findings and integrates the relationships between physiological state, oxygen atom exchange netween nitrite and water, and

isotopocule signatures. We believe this overview complements the detailed discussion and helps readers interpret the broader implications of our results.

No 39): P23 L20: perhaps again use 'suggest' transient activity instead of 'indicate'?

Changed.

Associate editor comment:

In addition to the reviewers' comments, I feel that your manuscript lacks an abiotic perspective, specifically the potential role of chemical reactions of nitrite leading to N_2O formation with isotopic signatures, particularly SP values, which range widely between canonical bacterial denitrification values and values found in canonical nitrification, fungal denitrification, and abiotic N_2O formation. Please consider the studies by Wei et al. (2017a,b; 2019).

We thank the Associate Editor for this helpful suggestion. We agree that acknowledging the broader context of abiotic nitrite reactions strengthens the discussion of N_2O isotopocule interpretation. While the present study focuses exclusively on physiological controls on isotopic signatures during bacterial denitrification, we have revised both the Introduction and the Conclusions to acknowledge that abiotic nitrite reactions, including chemodenitrification and reactions between nitrite and organic matter, can produce overlapping SP values.

1. Introduction (P2, L20):

"Bacterial denitrification typically yields SP values near 0 ‰, whereas fungal denitrification, hydroxylamine oxidation, and abiotic nitrite reactions, including chemodenitrification, can produce higher or more variable SP values (Toyoda et al., 2005; Frame & Casciotti, 2010; Ostrom et al., 2011; Jones et al., 2015; Toyoda et al., 2017; Wei et al., 2019; Yu et al., 2020). Recent work has further highlighted the importance of Fe(II)-mediated nitrite transformations and their potential contribution to abiotic N_2O formation under environmentally relevant conditions (Visser et al., 2022)."

2. Conclusion (P26, L8):

"Moreover, abiotic nitrite reactions, including chemodenitrification and reactions between nitrite and organic matter, have also been shown to produce a broad range of SP values overlapping those of biological pathways (Jones et al., 2015; Visser et al., 2022; Wei et al., 2019). Together, these findings further emphasize that N_2O isotopocules should be interpreted within their physiological and environmental context rather than as unique process-specific fingerprints."